Bayer HealthCare
Bayer Schering Pharma

Department: **GDD-GED-Toxicology** **GLP Report**

Report No.: AT06210

Test Item: **PES Vorstufe 2342**

Title: **LOCAL LYMPH NODE ASSAY IN MICE (LLNA/IMDS)**

Study No(s).: T9081299

Author(s): **Prof. Dr. H.-W. Vohr**

Study Completion Date: **Feb. 21, 2011**

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GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice as revised in 1997 (ENV/MC/CHEM(98)17) and with the revised German Principles of Good Laboratory Practice according to Annex I German Chemicals Act (Bundesgesetzblatt, Volume 2008, Part I, No 28, 1173-1184, issued July 11, 2008).

Prof. Dr. H.-W. Vohr
(Study Director)

Feb. 9, 2011
Date

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Quality Assurance Statement

Study No.: T9081299

Test Item: PES Vorstufe 2342

On the dates given below inspections were conducted by the Quality Assurance to ensure that no deviations exist that are likely to affect the integrity of this study.
The Quality Assurance Unit monitors the conduct of each study by study-based inspections or by process-based inspections of a similar type of study if the short-term nature of a study precludes inspection while it is in progress. Routine procedures and the equipment used in the relevant laboratory areas are inspected regularly and reports are made in accordance with current SOPs.

*(study plan amendments, if any, were duly audited and reported to the Study Director and Management)

Date of Audits / Inspections	**Phases Audited / Inspected**		**Date of Report to Study Director and Management**
Dec-02-2010	Study Plan *		Dec-02-2010
Nov-18-2010	process based	Preparation / Processing of Samples, Necropsy, Measurements, Sampling of Specimens, Weighing, Raw Data / Documentation	Nov-18-2010
Feb-18-2011	Main Report	1. Draft	Feb-18-2011
Feb-18-2011	Main Report	Final Draft	Feb-18-2011

The results of this study including the methods used have been checked on the basis of the current SOPs.
They have been correctly reported and the report reflects the raw data.
In case of a multi-site study audits at the test sites are presented in the QA Statement of the Principal Investigator's report (see appendix).

Quality Assurance Unit
Global R&D Quality, GLP-Mgmt.

Date: Feb-18-2011

Signature: 

Christina Kiedrowski

2 SIGNATURES

Study Director and Principal Expert in Immunotoxicology:	Prof. Dr. H.-W. Vohr	Feb. 21, 2011 Date

3 SUMMARY

The modified Local Lymph Node Assay (IMDS) was performed in 2010 on 24 female NMRI mice of the strain Crl:NMRI BR (6 animals/test item group and 6 control animals) to determine if there is any specific (sensitizing) or non-specific (irritant) stimulating potential of the test item PES Vorstufe 2342.
This study was carried out with NMRI mice from commercial breeder Charles River Germany, which is not the normally used breeder of the lab. Therefore, a concurrent control of 6 animals treated with Alpha Hexyl Cinnamic Aldehyde was included.

The study was conducted according to OECD Guidelines No. 429 and No. 406, EC Guideline 2004/73/EC (29th Adaptation of Guideline 67/548/EEC, B.42)/Health Effects Test Guideline and OPPTS 870.2600 (EPA) with the following test item concentrations:

Test item: 0 % (vehicle control), 2 %, 10 % and 50 %.
Positive control: 30 % Alpha Hexyl Cinnamic Aldehyde

The test item and the positive control were formulated in methyl ethyl ketone (MEK) to yield solutions.

Compared to vehicle treated animals there was an increase in cell counts in the mid and the high dose group. These increases are of no statistical significance. However, the "positive level" of index 1.4 [6, 8, 9] for the cell counts has been exceeded in both dose groups.

The "positive level" of ear swelling, which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10 % of the control values, has not been reached or exceeded in any dose group.
A slight statistically significant increase in ear weights had been determined for group 4 on day 4.

In conclusion, these results show that the test item PES Vorstufe 2342 has a moderate sensitizing potential in mice after dermal application of a 10 % concentration with an EC value of 3.71 %.

Therefore, the concentration of 2 % turned out to be the NOEL for the parameters investigated in this study with respect to skin sensitization.

These results are verified by the comparison with the results of the positive control group (Alpha Hexyl Cinnamic Aldehyde).

4 INTRODUCTION

A modified Local Lymph Node Assay (IMDS) was carried out in mice with the following test item:

PES Vorstufe 2342.

The modifications refer to the measurement of cell proliferation by cell counting instead of radioactive labeling. In addition, the acute inflammatory skin reaction is determined to discriminate specific from non-specific activation of immune competent cells in the draining lymph nodes [cf. 4, 6, 8, 9, 10, 23-25], as also recommended in the update of OECD TG 429.

The aim of these investigations was to establish whether there is any specific (sensitizing) or non-specific (irritant) stimulating potential of the test item PES Vorstufe 2342.

The investigations were carried out at the Bayer Schering Pharma AG, GDD-GED-Toxicology in 42096 Wuppertal, Germany.

The study plan, raw data and the final report are retained in the archives specified by Bayer Schering Pharma AG, Wuppertal, GDD-GED-IC-PM.
A retention sample of the test item is archived in the test item storage, Bayer Schering Pharma AG, Wuppertal, GDD-GED-IC-AHPM.

5 STUDY IDENTIFICATION AND RESPONSIBILITIES

5.1 Study identification

The laboratory study was carried out under number LLN10.156.

Study number:	T9081299
Experimental starting date:	December 6, 2010
Experimental completion date:	December 9, 2010
Study completion date:	see signature page
Sponsor:	Bayer MaterialScience AG, 51368 Leverkusen, Germany

5.2 Responsibilities

Head of HealthCare Toxicology:	Dr. F.-W. Jekat
Test facility management:	Dr. T. Steger-Hartmann
Study Director and Principal Expert in Immunotoxicology:	Prof. Dr. H.-W. Vohr
Head of Quality Assurance Unit:	Dr. A. Paeßens
Archiving of study data:	R. Zils

6 MATERIAL AND METHODS

The methods used in this study are in principle specified in guidelines (OECD 406, 1992; EPA guideline OPPTS 870.2600, Skin Sensitization, March 2003; CPMP/SWP/2145/00, 2001; updated OECD TG 429, 2010). According to these guidelines the so-called Local Lymph Node Assay (LLNA) is recommended for the assessment of skin sensitization as first-stage screening study or as a stand-alone test (updated OECD TG 429; OPPTS 870.2600).

The principle of the method had been published in 1989 [1], and a first collaborative validation study in 1991 [2]. In these first trials the stimulation of the lymph nodes, i.e. cell proliferation, was measured by ^{3}H-Thymidin incorporation. In 1999 the principle of the LLNA had been stated as valid alternative to guinea pig assays by the ICCVAM [3], although the need for further modifications was also noted.

A modification of the assay by measuring the cell counts instead of radioactive labeling provides comparable sensitivity [4, 5, 24, 25, 28], and has the advantage that the cell suspension can be further analyzed by different methods (flow cytometry, chemiluminescence responses, immunofluorescence) to gain an insight into mechanistic events [4-7]. A further modification was done by including the measurement of the ear swelling after treatment leading to a much more simplified and reliable assay (Integrated Model for the Differentiation of Skin reactions (IMDS), [8]). By comparing the specific immune reaction induced by the test item in the draining lymph nodes (LN; cell counts / LN weights) with the immediate unspecific acute skin reaction (ear swelling / ear weight) it is possible to discriminate the irritant potential from the sensitizing potential of the compound tested. International standards have been successfully determined using this modification [9, 24, 25]. Such modifications are also authorized in the Note of Guidance SWP/2145/00 of the CPMP (2001) and updated OECD guideline 429.
With respect to this simple discrimination between sensitizing and irritant local reactions comparable findings have been reported in the human patch test system [10].

6.1 Test item / positive control

6.1.1. PES Vorstufe 2342 (test item)

Test item:	PES Vorstufe 2342
Chemical Name:	Castor Oil, reaction product with Soybean Oil
Purity*:	100 % (not used for calculation)
Batch No.:	LB06603520
Approval:	until March 21, 2011
Physical state:	liquid, low viscous
Appearance:	colourless
Storage:	refrigerator, light protection

* This information was taken from the accompanying document

6.1.2. Alpha Hexyl Cinnamic Aldehyde (positive control)

Manufacturer:	Sigma Aldrich
Batch-No:	MKAA2596
Purity:	85 % (not used for calculation)
Approval:	until January 11, 2011
Physical state:	liquid
Appearance:	clear, yellow
Storage:	at room temperature

6.2 Animals

6.2.1. Selection of the species

SPF-bred female NMRI mice of the strain Crl:NMRI BR were used from commercial breeder Charles River Germany Sulzfeld, Sandhofer Weg 7, 97633 Sulzfeld, Germany. Animals of this strain have been used for years for toxicity studies at Bayer Schering Pharma AG. Mice of this strain have also been used for the intra-laboratory validation of the IMDS [8, 9], as well as an inter-laboratory validation study [24, 25].
Historical data on their physiology and spontaneous alterations are available. The health status of the strains is checked regularly at random for the most important specific infection pathogens, and the results of these tests are stored at Bayer Schering Pharma AG.

6.2.2. Adaptation

After their arrival, the animals intended for the study were allowed to adapt to the conditions of the animal room for at least 6 days and their state of health was monitored.

6.2.3. Health status

Only healthy animals showing no signs of disease were used in the study. The animals were not vaccinated or treated with antiinfectives either before their arrival or during the adaptation or study period. The females were nulliparous and nonpregnant.

6.2.4. Age and Body weight

The mice exhibited a weight of 27 – 33 grams at the beginning of the study. The age of the animals was 7 weeks.

6.3 Housing of the animals

6.3.1. Housing conditions

During the adaptation period up to 8 mice were housed together in conventional Makrolon® type III cages [11, 12], cages were changed at least twice a week. While during the study period the animals were single-housed in type II cages.

Low-dust wood shavings named Lignocel BK 8-15 supplied by Rettenmaier & Söhne, GmbH & Co, 73494 Rosenberg, Germany were used as bedding. At the instigation of the Laboratory Animal Services (LAS) the wood granulate was analyzed at random for contaminants. The relevant documents have been retained. The analytical results have not yielded evidence of any influence on the study objective.

6.3.2. Animal rooms

All the animals used in this study were kept in the same room. At times animals taking part in other toxicological studies were kept in the same room, but adequate spatial separation and appropriate organization of the working procedures ensured that animals could not be confused.

6.3.3. Cleaning, disinfection, and pest control

The animal room was cleaned once a week and disinfected at least once a month with Lysoform®d. Contamination of the feed and contact with the animals were excluded.
Pest control was not carried out in the animal room, but Killgerm Roach Traps which use no pesticides were placed in the animal room for cockroach control.

6.3.4. Environmental conditions

The environmental conditions in the animal room were standardized as follows:

Room temperature:	22 ± 2 °C
Relative humidity:	40 - 70 %
Light/dark cycle:	12 h/12 h, with artificial illumination
Air throughput:	About 10 changes per hour

Occasional deviations from these conditions occurred e.g. as a result of the cleaning of the animal room, but they had no apparent effect on the course of the study.

6.3.5. Diet

The feed, PROVIMI KLIBA SA 3883 maintenance diet for rats and mice (from Provimi Kliba SA, CH-4303 Kaiseraugst), and tap water (drinking bottles) were provided ad libitum.

The nutritive composition of PROVIMI KLIBA SA 3883 and the contaminant content of the standard diet were checked and analyzed routinely in random samples. The tap water was of drinking water quality [13].

The results of the feed and water analyses have been stored at Bayer Schering Pharma AG. The available data do not show evidence of any effects on the study objective.

Polycarbonate bottles with a capacity of about 300 ml (study period) or 700 ml (adaptation period) were used for drinking water [11, 12].

6.4 Methods

The method used has been described in the literature (see above). Unless internationally recognized standardized reference values or tests are available, the method used here must be viewed with this in mind. On the other hand there are enough peer reviewed data available confirming the validity of this method [reviewed in 3, 4-9], and an international catch-up validation study verified the reliability of this method (24, 25).

6.4.1. Methodological Reliability

The Local Lymph Node Assay Test methodology was checked for reliability in a test on female NMRI mice using Alpha Hexyl Cinnamic Aldehyde formulated in different vehicles (PEG 400, DAE 433, DMF, MEK, acetone/olive oil (4:1) and Cremophor EL/ physiological saline solution 2 % v/v) at concentrations of 3 %, 10 % and 30 %.
The sensitivity as well as the reliability of the experimental technique is thus confirmed by this study [26].
A similar check is done in regular intervals using one of the above mentioned vehicles in order to confirm the reliability of the method. The last reliability test using Alpha Hexyl Cinnamic Aldehyde formulated in acetone/olive oil (4:1) at concentrations of 3 %, 10 % and 30 % clearly showed the sensitizing potential of the test item [27].

6.4.2. Procedure

6.4.2.1. Grouping and identification of the animals

Six animals were placed in each group.
The animals were identified by cage labels giving the test item, the animal number, dose, sex, and the study number and marking of the tail immediately before autopsy.

6.4.2.2. Application schedule

	Ears
	Day 1-3
Group 1	Vehicle (MEK)
Group 2	2 % PES Vorstufe 2342 (in MEK)
Group 3	10 % PES Vorstufe 2342 (in MEK)
Group 4	50 % PES Vorstufe 2342 (in MEK)
Group 5	30 % Alpha Hexyl Cinnamic Aldehyde (in MEK)

6.4.2.3. Test item and the positive control formulation

6.4.2.3.1. Test item

The test item was formulated, once before application in MEK.
The formulations were visually described as solutions.
The stability of the test item in the vehicle was analytically verified for up to 4 days.

Results of the Stability of the test item in the formulation

Nominal value mg/ml	Theoretical value mg/ml	Normalized area units			Recovery as % of start value		
		start	after 2 hours	after 4 days	start	after 2 hours	after 4 days
10	9.982	100.2	95.3	93.9	100	95	94
500	500.072	98.0	96.0	97.9	100	98	100

Data taken from Study number: 2010/0087/20

6.4.2.3.2. Positive control

HCA was formulated immediately before each administration in MEK.
The formulations were visually described as solutions.
The stability of the HCA in the vehicle was analytically verified for up to 2 hours.

Results of the Stability of HCA in the formulation

Nominal value in %	Content in %		Content as % of nominal value	
	Start	After 2 h	Start	After 2 h
1	1.095	1.106	110	111
50	47.672	48.598	95	97

Data taken from F 7010916

6.4.2.4. Route of administration and dosage

The test item (described in Section 6.1) in the formulation (described in Section 6.4.2.3), the positive control in the formulation or the vehicle were applied as described in the application schedule (see 6.4.2.2), epicutaneously onto the dorsal part of both ears of the animals. This treatment was repeated on three consecutive days (d1, d2 and d3).

The volume administered was 25 µl/ear.

Based on our experiences with this test system, the known properties and due to the low viscosity of the test item the following concentrations were used: 0 % (vehicle control), 2 %, 10 % and 50 %. The highest concentration was set to 50 % (formulated in MEK) to avoid the formulation from dripping off the skin.
A concentration of 30 % was used for Alpha Hexyl Cinnamic Aldehyde as the positive control.

6.4.3. Investigations

6.4.3.1. Autopsies

The animals were anaesthetized by inhalation of carbon dioxide and sacrificed one day after the last application (day 4). The appropriate organs were then removed. Lymphatic organs (the auricular lymph nodes) were transferred into physiological saline (PBS).

6.4.3.2. LLN Weight and cell count determinations

The weight and cell count determinations were carried out by appropriate laboratory procedures. The weights of the lymph nodes were determined on a Mettler semiautomatic balance and stored in an IBM compatible PC. After crushing the lymph nodes through a sieve in a 12-well plate, the cell counts per ml were determined using a Multisizer 3® from Coulter Electronics. These data were also directly collected and processed by computer (Multisizer 3 software and Excel). Means, indices and standard deviations were calculated by an Excel data sheet.
A special BASIC program (GWBASIC compiler) was used to calculate means and standard deviations of the Lymphnodes' weights. Indices were calculated manually.

The so-called stimulation (or LLN-) index is calculated by dividing the absolute number of weight or cell counts of the substance treated lymph nodes by the vehicle treated ones. Thus, in case of no stimulating effect the index is always about 1.00 (+/- standard deviation), and the indices of vehicle treated animals are set to 1.00 (+/- standard deviation).

6.4.3.3. Ear Swelling

Before the first treatment and before sacrifice the thickness of both auricles of the animals was measured using a spring-loaded micrometer (Oditest, Dyer Company or Fa. Kroeplin). Means, indices and standard deviations of the ear swelling were calculated by an Excel data sheet.

6.4.3.4. Ear weight

On day 4 of the study the ear weight of the sacrificed animals was measured using a punch to take of a piece of every ear with a diameter of 8 mm. The weights were determined on a Mettler semiautomatic balance. Means, indices and standard deviations of the ear weights were calculated by an Excel data sheet.

6.4.3.5. Body weights

The body weights of the animals were recorded at the start and the end of the study (day 1 and day 4); cf. Appendix 11.4.

6.4.4. Statistics

When it was statistically reasonable, the values from treated groups were compared with those from the control group(s; vehicle) by a one-way analysis of variance (ANOVA) [15, 16] when the variances are considered homogeneous according to a homogeneity testing like Cochran`s test [17]. Alternatively, if the variances are considered to be heterogenous ($p \leq 0.05$), a non-parametric Kruskal-Wallis test has been used (Kruskal-Wallis ANOVA) at significance levels of 5 %. Two sided multiple test procedures were done according to Dunnett [18, 19] or Bonferroni-Holm [20], respectively. Outlying values in the LN weights were eliminated at a probability level of 99 % by Nalimov's method [21]. In addition, for the LLNA/IMDS the smallest significant differences in the means were calculated by Scheffe's method [22], which according to Sachs [17] can be used for both equal and unequal sample sizes.
In this method of statistical processing of measurements a large number of comparisons is made, and as a result of the multiple tests the overall probability of error is considerably greater than the p values suggest (increased number of false-positive results). On the other hand, the known methods of adjusting p values lead to an excessive increase in the number of false negatives. In view of these problems the biological and toxicological relevance is also taken into consideration in the evaluation of statistical significance.

For this reason, in the case of indices only the standard deviations between groups and difference analysis of the mean values were used in the evaluation of the biological relevance.

7 RESULTS

7.1 Stimulation indices (weight and cell counts; ear swelling and ear weight)

Based on results obtained in validation studies and general experiences with this test system (see Section 6 and 6.4) groups of mice were treated with vehicle, 2 %, 10 % or 50 % PES Vorstufe 2342 in MEK.

The NMRI mice did show an increase in the stimulation indices for cell counts (Appendix 11.1 and 11.2, 1.) in the mid and the high dose group, which is of no statistical significance, compared to control animals after application of the test item PES Vorstufe 2342. However, the "positive level", which is 1.4 for cell count indices, has been exceeded in both dose groups.

The "positive level" of ear swelling, which is $2x10^{-2}$ mm increase [8,9], i.e. about 10 % of the control values, has not been reached or exceeded in any dose group (Appendix 11.2, 2.).

A slight statistically significant increase compared to vehicle treated animals regarding ear weights was, however, detected in the high dose group. This increase may point to a slight irritant property of the test compound.

After treatment with Alpha Hexyl Cinnamic Aldehyde (group 5) the NMRI mice showed clear increases in the weights of the draining lymph nodes and in the stimulation indices for cell counts (Appendix 11.1 and 11.2, 1.) compared to control animals, which are of statistical significance. The "positive level", which is 1.4 for cell count indices has clearly been exceeded.

The "positive level" of ear swelling, which is $2x10^{-2}$ mm increase [8, 9], i.e. about 10 % of the control values, has been exceeded in the positive control group (Appendix 11.2, 2.). Statistical significant increases of the ear weights (Appendix 11.2, 3.) and ear swelling compared to control animals were also detected for the positive control group.

It has to be clarified that the "positive levels" mentioned above are exclusively defined for the NMRI outbreed mice used for this study [8, 9]. Such positive limits have to be calculated for each strain of mice individually [24, 25].

7.2 Body weights

The body weights of the animals were not affected by any treatment (Appendix 11.4).

8 DISCUSSION AND EVALUATION

A LLNA/IMDS was carried out in female NMRI mice after epicutaneous application of a formulation containing 0 %, 2 %, 10 % or 50 % of the test item PES Vorstufe 2342 for 3 consecutive days onto both ears of the animals.

This study does point to a specific immunostimulating (skin sensitizing) potential of the test item.

This applies to NMRI mice, for weight and cell counts of the draining lymph nodes as well as ear swelling and ear weight indices evaluated after application of PES Vorstufe 2342.

After treatment with PES Vorstufe 2342 there was a clear increase compared to control animals regarding the stimulation indices for cell counts in the mid and the high dose group, which is of no statistical significance. The "positive level", which is 1.4 for cell counts, has been exceeded in both dose groups.

A sensitizing potential can be assumed from the increases in cell proliferation in the draining lymph nodes. On the basis of our experiences using this method the "positive level" had been set to an increase in cell count index by 0.4 (i.e. index ≥ 1.4), which has been exceeded in the mid and the high dose group. Differentiation indices (DI) calculated according to our publications [8, 9] which is the quotient of the relative lymph node reaction divided by the relative acute skin reaction were > 1 for the mid and the high dose group of the test item, i. e. 2.13 and 2.42, respectively. These DI values do also point to a skin sensitizing potential of the test item.

The "positive level" of ear swelling which is $2x10^{-2}$ mm increase [8, 9], i.e. more than 10 % increase in index, has not been reached or exceeded.

The EC 1.4 value calculated is 3.71 % for this test item. In accordance with the classification proposed in the Technical Report No. 78 of the ECETOC [29] this value corresponds to a moderate skin sensitizer.

Taken together, a specific activation of the cells of the immune system via dermal route was determined after application of 10 % PES Vorstufe 2342 by the method used. Thus, **PES Vorstufe 2342** has to be classified as a moderate skin sensitizer.
Therefore, the concentration of 2 % turned out to be the NOEL for the parameters investigated in this study.

These findings were verified by the results obtained with the positive control compound Alpha Hexyl Cinnamic Aldehyde.

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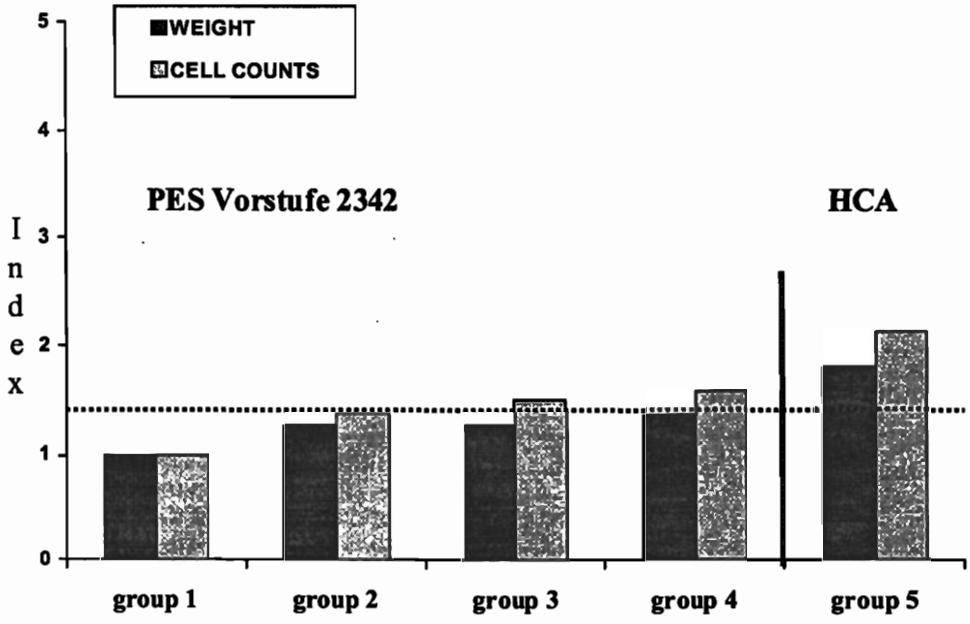
10 ABBREVIATIONS

approx.	approximately
A/OO	acetone/olive oil (4:1)
B	B cell
CC	cell counts
^{o}C	degrees centigrade
cf	confer
d	day
DAE 433	dimethylacetamide (40 %), acetone (30 %) and ethanol (30 %)
DMF	dimethylformamide
DMSO	dimethylsulfoxide
FACScan	Fluorescence Activated Cell Scanner
HCA	Alpha Hexyl Cinnamic Aldehyde
h	hour
i.e.	id est
IMDS	Integrated Model for the Differentiation of chemical-induced Skin reactions
kg	kilogram
LN	lymph node
LLN	local lymph node
LLNA	local lymph node assay
MEK	methyl ethyl ketone
mg	milligram
ml	milliliter
MO	macrophage
NaCl	sodium chloride
no.	number
PBS	phosphate buffered saline
PEG 400	polyethylene glycol 400
Pluronic/ NaCl	Pluronic PE 9200/ 0.9 % NaCl solution, 1 % v/v
rel.	relative
SD	standard deviation
T	T cell
Veh.	vehicle
v/v	volume/volume
w/w	weight/weight
w/v	weight/volume

11 APPENDIX

11.1 Bar charts (weight and cell count) for the LLNA



Group definition see 6.4.2.2.

11.2 Tabular summary of the LLNA/IMDS results

1. Direct LLNA (NMRI mice, female, 6 animals/group)

Groups	**Weight index** (index of mean +/- SD in %)	**Cell count index** (index of mean +/- SD in %)
Gr. 1	**1.00** +/- 15.74	**1.00** +/- 31.01
Gr. 2	**1.27** +/- 28.95	**1.37** +/- 31.76
Gr. 3	**1.26** +/- 31.69	**1.51** +/- 42.37
Gr. 4	**1.37** +/- 24.20	**1.58** +/- 24.98
Gr. 5	**1.80**↑ +/- 11.73	**2.12**↑ +/- 22.48

2. Ear swelling (NMRI mice, female, 6 animals/group, in 0.01 mm)

Groups	day 1 (mean +/- SD in %)	day 4 (mean +/- SD in %)	Index day 4
Gr. 1	**17.75** +/- 4.88	**18.17** +/- 5.16	1.00
Gr. 2	**17.67** +/- 3.69	**18.25** +/- 2.48	1.00
Gr. 3	**17.58** +/- 4.51	**18.33** +/- 5.85	1.01
Gr. 4	**17.42** +/- 2.96	**18.67** +/- 5.75	1.03
Gr. 5	**17.75** +/- 3.50	**21.08**↑ +/- 8.92	1.16

↑ = statistically significant increase ($p \leq 0.05$)

3. Ear weight (NMRI mice, female, 6 animals/group, in mg per 8 mm diameter punch)

Groups	day 4 (**mean** +/- SD in %)	Index day 4
Gr. 1	**11.73** +/- 8.25	1.00
Gr. 2	**11.78** +/- 6.69	1.00
Gr. 3	**12.10** +/- 7.81	1.03
Gr. 4	**13.05**↑ +/- 9.26	1.11
Gr. 5	**15.53**↑ +/- 12.18	1.32

↑ = statistically significant increase ($p \leq 0.05$)

11.3 Individual cell counts

Cell counts [Thousand cells / mL cell suspension] in auricular lymph nodes* on study day 4 after daily epicutaneous application of a 0, 2, 10 or 50% solution of PES Vorstufe 2342 or Alpha Hexyl Cinnamic Aldehyde in methyl ethyl ketone for 3 days onto the ears of female NMRI mice (6 / treatment group) in a Local Lymph Node Assay

Treatment group	Animal no.	Cell count [Thousand cells / mL lymph node suspension]* Repeat determination	Arithmetic mean	Group mean	SD	SD [%]	Cell count index**
Vehicle control (Group 1)	1	8503 8341	8422	7642.17	2369.73	31.01	1.00
	2	10834 11053	10943.5				
	3	8226 8396	8311				
	4	6580 6606	6593				
	5	7783 7844	7813.5				
	6	3776 3764	3770				
Group 2	7	8472 8339	8405.5	10478.25	3327.55	31.76	1.37
	8	10382 10846	10614				
	9	14288 14307	14297.5				
	10	5858 5987	5922.5				
	11	14204 14352	14278				
	12	9331 9373	9352				
Group 3	13	11019 10997	11008	11541.58	4890.12	42.37	1.51
	14	13337 13466	13401.5				
	15	20360 20407	20383.5				
	16	9683 9511	9597				
	17	7543 7624	7583.5				
	18	7271 7281	7276				
Group 4	19	12492 12603	12547.5	12109.50	3024.87	24.98	1.58
	20	11390 11468	11429				
	21	16756 18497	17626.5				
	22	9106 9062	9084				
	23	12178 12231	12204.5				
	24	9630 9901	9765.5				
Group 5	25	11571 11547	11559	16226.33	3648.48	22.48	2.12
	26	15212 15473	15342.5				
	27	22224 22506	22365				
	28	14152 13847	13999.5				
	29	17107 17096	17101.5				
	30	17000 16981	16990.5				

* : The right and left auricular lymph node of each animal was obtained, crushed trough a sieve and dispersed in 2 mL phosphate buffered saline. An aliquot of this sample was taken for a repeated determination of the cell count per mL of this cell suspension. The cell count of every animal is the calculated mean of these two measurements.

** : Cell count index: mean cell count of the animals of a treatment group divided by the mean cell count of the vehicle control group. The Cell count index for the vehicle control group is 1.

11.4 Body weights

Animal-No.	Body weight in g	
	Day 1	**Day 4**
group 1		
1	29	30
2	31	32
3	28	27
4	30	29
5	31	29
6	33	32
Mean	**30.3**	**29.8**
group 2		
7	30	31
8	29	28
9	31	32
10	31	32
11	30	29
12	29	29
Mean	**30.0**	**30.2**
group 3		
13	30	31
14	29	29
15	28	27
16	29	29
17	30	31
18	29	30
Mean	**29.2**	**29.5**
group 4		
19	28	28
20	29	28
21	28	28
22	28	29
23	31	30
24	28	28
Mean	**28.7**	**28.5**
group 5		
25	31	32
26	28	29
27	27	26
28	29	29
29	30	29
30	28	27
Mean	**28.8**	**28.7**